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(54) Title: **A BACTERIAL STRAIN, PROCESSED PLANT EXTRACTS AND PROBIOTIC COMPOSITIONS FOR HUMAN AND VETERINARY USE**

(57) Abstract: A formulation comprising at least one volatile fraction (VF) of a plant extract, the volatile fraction being prepared by steam distillation of the plant extract under reduced pressure and at a bath temperature not exceeding 38 °C. The extract may be obtained from the leaves, stems, roots or fruit of the plant. The plant may be a vegetable or herb, such as parsley, mint or dill. The vegetable may be soy bean, alfalfa, garlic, beet and cabbage. The formulation may further comprise propolis or other beehive product.

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A BACTERIAL STRAIN, PROCESSED PLANT EXTRACTS AND PROBIOTIC COMPOSITIONS FOR HUMAN AND VETERINARY USE

FIELD OF THE INVENTION

The present invention relates to a non-pathogenic probiotic microorganism, a formulation comprising an aqueous solution of a volatile fraction (VF) prepared from the extract of at least one plant, and a probiotic composition comprising said probiotic microorganism or said formulation comprising said probiotic microorganism. The formulations of the invention are capable of sustaining the viability of the probiotic microorganism suspended therein for at least one year, under refrigeration. In addition, the formulations of the invention have an enhancing effect on weight increase in animals. The probiotic microorganism and compositions of the invention have beneficial and therapeutic activity in the gastrointestinal (GI) tract. Further, the invention relates to the process for preparing the formulations and the probiotic compositions according to the invention and to their different uses.

BACKGROUND OF THE INVENTION

Under normal conditions, the gastrointestinal (GI) tract microflora contributes significantly to the health and well being of an individual. It is well known that the microflora is a complex and diverse population, which may have both beneficial and harmful effects on the individual. In general, the foetus *in utero* is sterile, but on passage through the vagina during birth it acquires microorganisms resulting in the formation of a gut microflora. The final indigenous gut microflora which stabilizes in the gut is a very complex collection of over one thousand microorganisms, consisting of 400 different types of bacteria [Fuller R.J. Applied Bacteriology 66:365-378 (1989)]. The composition of the flora is determined by host and microbial factors, and although there are a lot of bacteria which can survive and grow in the GI tract, there are many which cannot. In addition, the surviving organisms have to avoid the effect of peristalsis which normally flushes out the bacteria with the food. This may be achieved by the bacteria immobilizing themselves by

attachment to the gut wall, or by growing at a rate that is faster than the rate of removal by peristalsis.

The microflora protects the individual from infections caused by pathogens. This phenomenon has been described under such names as 'bacterial antagonism', 'bacterial interference', 'barrier effect', 'colonization resistance', 'competitive exclusion' and many others.

This protective flora is very stable. However, it is less effective in the young, elderly and the compromised patient. Further, it can be influenced by certain dietary and environmental factors, the three most important being excessive hygiene, antibiotic therapy and stress.

Under conditions where the balance of the gut microflora is adversely affected, probiotics become of potential value in restoring the gut flora and enabling the individual host to return to normal.

Probiotics are a class of microorganisms defined as live microbial organisms that beneficially affect the animal and human hosts. The beneficial effects include improvement of the microbial balance of the intestinal microflora or improving the properties of the indigenous microflora. The beneficial effects of probiotics may be mediated by a direct antagonistic effect against specific groups of organisms, resulting in a decrease in numbers, by an effect on their metabolism or by stimulation of immunity. Probiotics may suppress viable counts of an undesired organism by producing antibacterial compounds, by competing for nutrients or for adhesion sites. Further, they may alter microbial metabolism by increasing or decreasing enzyme activity or they may stimulate the immune system by increasing antibody levels or increasing macrophage activity.

The present invention is intended to provide a novel non-pathogenic probiotic microorganism derived from *E. coli*, a formulation comprising aqueous solution of a volatile fraction (VF) prepared from the extract of at least one plant, and a

probiotic composition comprising the probiotic organism suspended in said formulation.

WO95/16461 describes a probiotic or composition of anaerobic bacteria effective in controlling or inhibiting *Salmonella* colonization of domesticated animals. The probiotic includes populations or cultures of 29 substantially biologically pure bacteria, *inter alia*, *E. coli*. However, the suppression of the pathogen by the probiotic or compositions described in this publication require the combined action of a large number of bacterial strains.

WO97/35596 describes the administration of a freshly prepared probiotic mixture obtained by mixing a powder containing *Lactobacillus reuteri*, *Lactobacillus acidophilus* and *Bifidobacterium infantis* with a liquid. The mixture is described to be effective in preventing infectious diarrhea or diarrhea caused by antibiotic therapy in humans. The freeze-dried live bacteria are, however, in anabiotic state. The need to wet the microorganism before administration, in order to reinstate its vitality, is a disadvantage, since normally many bacteria do not survive the re-hydration. Moreover, the surviving organisms are not immediately metabolically active, and cannot survive the extreme, acidic conditions of the stomach.

Preservation of viability and conservation of the activity of probiotic organisms by their formulation is the issue of numerous publications. WO98/26787 describes the enhancement of a resident population of lactic acid-producing microorganisms, preferably *lactobaccillii*, in the GI tract of an animal by providing the same with β -glucan, optionally in combination with prebiotic and/or probiotic microorganisms.

WO97/34591 also describes the enhancement of resident population of microorganisms, or the suppression of the undesired resident population at a selected site of the GI tract of an individual, by providing the individual with a selected modified or unmodified starch or mixtures thereof, which act as carrier for one or more probiotic microorganisms and as a growth or

maintenance medium for the microorganisms. The probiotic elements are bound to the carrier in a manner so as to protect the microorganisms during passage to the large bowel or other regions of the GI tract.

It has been unexpectedly found that a single species of a non-pathogenic probiotic microorganism derived from *E. coli* is alone capable of restoring normal GI flora of man and of a variety of animals and birds. It has also been surprisingly found that this microorganism could be preserved for long time, in a viable and metabolically active form, in a formulation comprising water solution of volatile fraction/s of various plant extracts.

The probiotic composition comprising the probiotic organism in the said formulation was found to be effective in the treatment and prevention of various gastrointestinal disorders.

It has further been unexpectedly found that the formulation *per se* is effective as a body weight gain enhancer and an immuno-stimulator in mammals and birds.

SUMMARY OF THE INVENTION

The present invention relates to a non-pathogenic probiotic microorganism, namely *E. coli* strain BU-230-98 (ATCC Deposit No. 202226).

The present invention also relates to a formulation comprising an aqueous solution of a volatile fraction (VF) prepared from the extract of at least one plant. The volatile fractions of the plant extracts are prepared by steam distillation of said plant extract under reduced pressure and at a bath temperature not exceeding 38°C.

The plant matter utilized in the formulations of the invention may be any suitable fruit, vegetable, leaf, stem or root, and is preferably apple, citrus fruits, soy, beet, parsley, mint, dill, garlic, alfalfa, and cabbage.

The formulations of the invention may be used as veterinary preparations and food/feed additives, having enhancing activity on body weight gain. The formulations of the invention may also be used as suspension media for microbiotic microorganisms. Under refrigeration, the formulations of the invention can sustain the viability of the probiotic microorganisms suspended therein for at least one year. The term food/feed additive as used herein is to be taken to mean food additive or nutritional additive for human use or a feed additive or nutritional additive for use with livestock and poultry, as well as other domesticated animals.

The invention further relates to probiotic compositions comprising microbiotic microorganism/s suspended in the formulations of the invention. The microbiotic compositions of the invention have beneficial biological activity and therapeutic effects in the GI tract. Like the formulations of the invention, also the present probiotic compositions may be used as food/feed additives, as defined above.

The food/feed additive of the invention, be it the formulation or composition of the invention, may have numerous applications, *inter alia*, preventing or treating gastro-enteric infections, treating or preventing infectious diarrhea, chronic diarrhea or diarrhea caused by infection, by antibiotic or chemo-therapy, treating enterocolitis, effectively restoring the GI microflora, treating dyspeptic symptoms, increasing body weight gain, stimulating the immune system in a subject; alleviating lactose intolerance in subjects suffering therefrom, normalizing GI food metabolism, treating constipation and/or reducing blood cholesterol levels.

It yet a further aspect, the invention relates to a process for preparing a volatile fraction of a plant extract, which process comprises the steps

of:-(a) grinding a plant matter to obtain a biomass; (b) mixing the plant biomass obtained in step (a) with water at a certain proportion, preferably, at weight proportion of 3 parts water to 1 part of the plant biomass and stirring the same for at least 2 hours at ambient temperature; (c) distilling the mixture obtained in the step (b) under reduced pressure and at a bath temperature not exceeding 38°C; (d) collecting the volatile fraction which may be further diluted in a suitable buffer. This fraction itself may constitute a formulation of the invention.

The invention relates also to a process for the preparation of the formulations of the invention, by mixing several volatile fractions of various plant extracts and optionally mixing them with water, and suitable additives, adjuvants or carriers.

The invention further provides a process for preparing probiotic compositions, by suspending at least one viable probiotic microorganism having a beneficial biological activity and/or therapeutic activity in the GI tract, in the volatile fraction obtained by the said process of the invention or in a mixture of several such volatile fractions.

Finally, the invention relates to the use of at least one volatile fraction (VF) of a plant extract in the preparation of the formulations, food/feed additives and probiotic compositions of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a probiotic microorganism being a non-pathogenic bacterium derived from *E. coli*, having a beneficial physiological and/or therapeutic activity in the GI tract and deposited at the ATCC under deposit No. 202226 and at DSMZ under deposit No. 12799.

The present invention further relates to a formulation comprising at least one volatile fraction (VF) of a plant extract, the volatile fraction being prepared by

distillation of said plant extract under reduced pressure and at a bath temperature not exceeding 38°C.

The plant matter from which the volatile fraction may be obtained may be any suitable plant part such as fruit, leaf, stem or root. Many plants are suitable as a source for the volatile fractions, for example apple, citrus, soy bean, beet, cabbage, garlic and alfalfa, as well as herbs such as parsley, mint and dill. The formulations of the invention may optionally further comprise a suitable amount of a volatile fraction of an apicultural product such as honey, propolis or other beehive product, which may be prepared in the same manner as described herein for obtaining volatile fractions from plant extracts.

As will be described in more detail in the following Examples, the formulation itself may be used as a food/feed additive. It has been found by the inventors to have a weight gain enhancing activity and constitutes an aspect of the invention. According to a particular embodiment of this aspect of the invention, the animal weight gain enhancing formulation or feed additive comprises distilled water and volatile fractions of alfalfa, soy beans, beet and dill, preferably at a volume ratio of the volatile fractions of 2:8:1:4.

In a further aspect, the invention relates to a probiotic composition comprising the said formulation of the invention and at least one viable probiotic microorganism having a therapeutic or beneficial biological activity in the GI tract.

A particular advantage of the probiotic composition of the invention stems from the fact that it is a liquid preparation. Being under biologically active conditions, the formulation serves also as a supportive medium for living bacteria, as opposed to lyophilized formulations where the bacteria are in an anabiotic condition. As a result, the probiotic composition of the invention is active immediately following oral administration, beginning with the upper portion of the GI tract, where primary effects of the majority of intestinal pathogens take place, causing development of adverse gastro-enteric

syndromes. The probiotic compositions of the invention may also be used as body weight-increasing preparations or food/feed additives.

It should be noted that bacteria other than such belonging to the strain BU-230-98 (ATCC Deposit No. 202226, also deposited at the DSM under No. 12799), may be used with the formulation of the invention. Such bacteria have a very broad spectrum of antagonistic activity. They also belong to the same phylogenetic group of the majority of intestinal pathogens and share the same systems of survival. Therefore, the suppression and exclusion of intestinal pathogens may include many different mechanisms, for example, secretion of antagonistic material, competition for utilization of nutrients and competition for adhesion receptors. Thus, any non-pathogenic bacteria which comply with these criteria, may be used in the probiotic compositions of the invention.

According to a further aspect of the invention, the probiotic compositions of the invention may be used for preventing or treating gastro-enteric infections. Term 'gastro-enteric infection' is to be taken to mean any infection caused an enteric pathogen, including, *inter alia*, Gram negative and Gram positive bacteria. By improving the general balance and health of the GI tract, the formulations and probiotic compositions of the invention may be instrumental in prophylaxis of also GI infections caused by yeast, viruses and protozoa.

The term 'therapeutically effective amount' or 'effective amount' for purposes herein is the amount determined by such considerations as are known in the art. The amount must be sufficient to enable the efficient restoration of the GI microflora thus leading to the normalization of the function of the GI tract.

In a particular embodiment, the probiotic composition for preventing the development of gastro-enteric infections comprises the formulation of the invention made from distilled water and volatile fractions of alfalfa, soy beans, beet, dill and mint as defined herein, at a volume ratio of, e.g., 5:1:5:15:1, and a therapeutically effective amount of a probiotic bacteria such as *E. coli* ATCC Deposit No. 202226 (identical with DSM 12799).

A specific example for an gastro-enteric infection is that caused by *Salmonella* and the invention is of particular advantage in preventing or treating gastro-enteric infections caused thereby.

Further, the food additive or formulation of the invention, alone or in combination with an effective amount of a probiotic microorganism, such as the *E. coli* strain ATCC Deposit No. 202226 (identical with DSM 12799), may be used for treating or preventing infectious diarrhea, chronic diarrhea or diarrhea caused by antibiotic or chemo-therapy.

According to a further specific embodiment of the invention, such a probiotic composition for treating infectious diarrhea may comprise distilled water and volatile fractions of alfalfa, soy beans, beet, dill and mint at a volume ratio of, e.g., 5:1:5:15:1, and a therapeutically effective amount of the ATCC No. 202226 (DSMZ 12799).

The infectious diarrhea may be caused by numerous factors, for example, by a microorganism selected from *C. difficile*, *Salmonella*, particularly *S. Shigella*, *Campylobacter*, *E. coli*, *Proteus*, *Pseudomonas*, *Clostridium*, enteric *Staphylococcus*. These are but few of many infecting agents.

Yet further, the probiotic compositions of the invention may be used for effectively restoring the GI microflora in a subject in need of such treatment which leads to the normalization of the function of the GI tract. Such compositions may include, for example, distilled water and volatile fractions of alfalfa, soy bean, beet, dill, mint, parsley and cabbage, preferably at a ratio of volatile fractions of, e.g., 1:5:5:2:2:1, and a therapeutically effective amount of the probiotic bacteria ATCC Deposit No. 202226 (DSM 12799).

Other purposes for which the probiotic compositions of the invention, comprising at least one probiotic having a therapeutic effect in the GI tract, include alleviation of lactose intolerance in subjects suffering from lactose intolerance, treatment of enterocolitis, treatment of constipation, for reduction

of cholesterol levels in the blood, for treatment of dyspeptic symptoms, and/or for stimulation of the immune system in subjects suffering from an immune system disorder, which may be an immune disorder caused by immuno-suppressive therapy.

In a different aspect, the invention relates to a process for preparing a volatile fraction of a plant extract, which process comprises the steps of:- (a) grinding plant matter to obtain a plant biomass; (b) mixing the plant biomass obtained in step (a) with water at a weight proportion of 3 parts water to 1 part of the plant biomass and stirring the same for at least 2 hours at ambient temperature; (c) distilling the mixture obtained in step (b) under reduced pressure and at a bath temperature not exceeding 38°C; and (d) collecting the volatile fraction obtained from said steam distillation, which fraction may further be diluted in a suitable buffer.

The volatile fraction of the invention may be mixed with water to give the food/feed additive or formulation of the invention. The food/feed additive or formulation of the invention may also be prepared by mixing more than one plant volatile fraction obtained by the process of the invention. This mixture may be further mixed with water.

The volatile fractions may be prepared from any suitable fruit, vegetable, leaf, stem or root of a plant. The plant can be, for example, apple, citrus fruit, soy bean, beet, garlic, cabbage or alfalfa, or a herb such as parsley, mint or dill. It should be noted that when appropriate, the formulation may further comprise volatile fractions from apicultural products such as honey or propolis or other beehive products. These volatile fractions may be prepared in the same manner of the plant extract volatile fractions.

According to the process of the invention, the distillation step is preferably carried out under reduced pressure of 5-10 mbar.

As indicated hereinbefore, the food/feed additive or formulation of the invention may be further combined with at least one probiotic agent, to give the probiotic compositions of the invention. Thus, the process of preparation of the invention may also further comprise the step of suspending at least one viable probiotic microorganism having a therapeutic activity in the GI tract in the volatile fraction obtained in said step (d) or in a mixture of such volatile fractions obtained as described above.

According to a particular process of the invention, the probiotic microorganism suspended may be the novel bacterium of the invention, derived from *E. coli* and deposited at the ATCC under Deposit No. 202226 (and at DSM under deposit No. 12799).

Finally, the invention relates to the use of a volatile fraction (VF) of a plant extract in the preparation of a food/feed additive, wherein the VF is prepared by steam distillation of said plant extract or from apiculture product extract such as honey or propolis at a bath temperature not exceeding 38°C.

The present invention is defined by the appended claims, the contents of which are to be read as included within the disclosure of the specification, and will now be described in more detail on hand of the following examples.

While the foregoing description describes in detail only a few specific embodiments of the invention, it will be understood by those skilled in the art that the invention is not limited thereto and that other variations in composition may be possible without departing from the scope and spirit of the invention herein disclosed.

EXAMPLES

Example 1

Preparation of the "volatile fraction" of plant extracts or extracts from apiculture products

Fresh vegetables obtained from commercial markets were thoroughly washed by tap water, chopped and finely ground in an industrial blender. Distilled water was added to the vegetable biomass at the proportion of 3 parts water to 1 part of the vegetable mass by weight and left under stirring for at least 2 hours at ambient temperature.

The mixture was then transferred into the evaporating flask of a rotatory evaporator and was evaporated at the reduced pressure (5-10 mbar) under such conditions that the temperature of the water bath did not exceed 38°C, and that of the condenser inlet was 2-5°C. About 2.5 l of the condensate (volatile fraction) were collected per one kg of plant biomass. This material could be preserved under refrigeration for at least 6 months without losing its properties.

The same procedure may be carried out, replacing the plant material with apiculture products such as honey or propolis.

Usually, each "volatile fraction" was prepared from a single kind of vegetable and used in different dilution to prepare different mixtures for various purposes.

Example 2

Growth of the probiotic organism *E. coli* ATCC Deposit No. 202226

The probiotic organism *E. coli* (deposited at the ATCC under No. 202226 on May 3, 1999 and at the DSM under No. 12799 on 4 May 1999) was grown in the following medium (g/l): (NH₄)₂SO₄ 5, KH₂PO₄ 13, Na₂HPO₄ 13, MgCl₂ 3,

CaCl₂ 0.3, yeast extract 10, Soy peptone 10, glucose 5. Additional nutrients (yeast extract 1, Soy peptone 2.5, glucose 90) were continuously added following the growth of the culture in such a way that the glucose concentration in the fermentation broth was kept at the level of 2 g/l. The pH of the fermentation broth was kept neutral by the continuous addition of 4N NH₄OH. Culturing was carried out at 30°C in a standard fermentation vessel with aeration of 0.5 vvm for 16 hours, when the growth became confluent. This procedure resulted in 10¹⁰-10¹¹ cells/ml. The *E. coli* cells were harvested by centrifugation, resuspended in saline and re-precipitated. The microbial biomass could be kept in the refrigerator for 48 hours without losing viability.

Example 3

Preparation of food/feed additive formulation for the accelerated increase of body weight in birds and animals

The mixture contained (ml/l) volatile fractions of: alfalfa 50, soy beans 200, beet 25 and dill 100. The balance was made up by the distilled water.

Example 4

Preparation of the food supplement for the normalization of the function of the human GI tract

The mixture contained (ml/l) volatile fractions of: alfalfa 50, soy beans 10, beet 50, dill 50, mint 20, parsley 20 and cabbage 10. The balance was made up by distilled water. *E. coli* (ATCC 202226) cells (Example 3) were suspended in the mixture at the cell concentration of 10⁷ cells/ml. NaCl (4-10 g/l) may be optionally added for the improvement of taste.

Example 5

Preparation of the feed additive formulation for the prophylaxis of GI infections in birds and animals

The mixture contained (ml/l) volatile fractions of: alfalfa 50, soy beans 10, beet 50, dill 150 and mint 10. The balance was made by distilled water. *E. coli* (ATCC 202226) cells (Example 2) were suspended in the mixture at a cell concentration of 10^7 cells/ml. NaCl (4-10 g/l) may be optionally added for the improvement of the taste.

Example 6

Antagonistic activity of *E. coli* (ATCC 202226) against *Salmonella typhimurium* (ATCC 14028)

Petri plates containing Modified Brilliant Green Agar, a selective growth medium for *Salmonella*, were inoculated with *S. typhimurium*. A 9 mm diameter well was made in the agar. A volume of the food supplement (Example 4) was deposited in each well, and the plates were incubated for 24 h at 35°C. The same was repeated, but instead of the food supplement, the fluid obtained by its filtration through a microbiological filter membrane (pore size of 0.45 µm) was deposited in the well.

Around each well containing the feed additive an inhibition zone (10-17 mm) devoid of *S. typhimurium* colonies was observed. No inhibition zone was observed around the wells containing the filtrate free of the probiotic organism.

Example 7

Antagonistic activity of *E. coli* ATCC 202226 as well as of its parent strain *E. coli* M-17 against *Shigella* sps

Cultures of *S. flexneri*, *S. sonnei*, *E. coli* (ATCC 202226) and M-17 were grown separately on the Nutrient Agar for 18-20 h at 37°C. All the cultures were harvested in saline and diluted to the optical density of 10 Klett units. Aliquots of the diluted cultures of *Shigella* species (1 ml) alone or in combination with the diluted culture of *E. coli* (ATCC 202226) (1 ml) were seeded in

ventilation-cup test tubes containing Nutrient Broth (5 ml). The tubes were incubated for 24 h at 37°C. The number of colony-forming units (CFU) of the pathogens and of *E. coli* (ATCC 202226) was determined by plating the cultures on Nutrient agar. The CFU numbers of two *Shigella* species in the pure culture and in mixed cultures with both probiotic *E. coli* species are shown in Table 1.

Table 1

Probiotic Organism	Growth of <i>Shigella</i> CFU/ml	
	<i>S. flexneri</i>	<i>S. sonnei</i>
<i>E. coli</i> ATCC 202226	$<5 \times 10^4$	$<5 \times 10^4$
<i>E. coli</i> M-17	1×10^6	2×10^6
Pure <i>Shigella</i> culture	6×10^6	2×10^6

Example 8

Application of the food supplement (Example 4) and of Colibacterin (dry formulation of *E. coli* M-17) in hospitalized gastroenteritis patients

A group of 60 patients that developed gastroenteritis following hospitalization was randomly divided into 3 sub-groups with a similar distribution of sex, age and the severeness of gastroenteritis symptoms. All patients received normal supportive treatment, rehydration, vitamins, etc. The severity of condition did not require treatment with antibiotics. The first group of 20 patients received 10 ml of the food supplement (Example 5) 3 times daily 30 min before the meals for 7 days. The second group of the same number received Colibacterin

(Colibacterinum siccum) as recommended by the producer (one dose twice a day 30 min before the meal) and the third group received no supplement at all. The onset of normalization (in days) of the symptoms of gastroenteritis in all groups were recorded and are shown in Table 2.

Table 2

Symptoms	Onset of the normalization of the symptom, days		
	Food supplement	Colibacterin	Control
Body temperature	2.8±0.2	2.7±0.2	3.7±0.2
Intoxication	2.5±0.1	3.6±0.1	4.6±0.1
Abdominal pain	3.3±0.2	5.1±0.2	6.1±0.2
Diarrhea	1.8±0.2	3.4±0.2	4.4±0.2

Colibacterin (Colibacterinum siccum) is the freeze-dried preparation of live *E. coli* M-17 produced by BIOMED Ltd., Moscow, Russia, and recommended for use against diarrhea [Vidal Handbook: Pharmaceutical preparations in Russia (N.B. Nikolaeva, B.P. Alperovich and V.N. Sovinov, Eds.) AstraPharmService, 1997, Moscow, p. 275].

Example 9

Application of the food supplement (Example 4) in patients with acute GI infections

Patients with severe GI infections of various etiologies: salmonellosis, escherichiosis, shigellosis, staphylococcal infections and food intoxications of unknown etiology. In all patients the hospitalization was indicated by an acute onset of the disease and appearance of acute gastroenteritis. The total of 186

patients were treated with the food supplement and a similar group of 102 patients received standard treatment.

The onset of normalization (in days) of the symptoms of gastroenteritis in all groups were recorded and are shown in Table 3.

Table 3

Symptoms	Onset of normalization, days	
	Study group	Control
Fever	1.2±0.3	2.7±0.3
Weakness	1.6±0.2	2.9±0.3
Abdominal pain	1.5±0.2	2.4±0.3
Diarrhea	2.3±0.2	4.7±0.4
Days in bed	4.8±0.3	6.7±0.4

In a separate study a group of 30 patients with intestinal typhoid was treated with the food supplement. In 80% of patients the symptoms of disease disappeared within 3 days. Only in 3 cases the supplement treatment was stopped due to the development of more severe chronic colitis.

Example 10

Applications of the food supplement (Example 4) in patients with GI disorders caused by antibiotics

Patients with severe GI disorders were divided in 2 groups. Group I contained 48 patients with peptic ulcer disease who developed GI disorders after

antibiotic treatment against *H. pylori*. Group II contained 22 patients that developed GI disorders following antibiotic treatment of pneumonia.

The food supplement (5 ml) was given 3 times a day before meals for 7 days. In both groups symptoms of diarrhea disappeared in 2-3 days in all the patients. After the administration of the supplement, complete normalization of intestinal microflora was observed in 84.5% of the patients. It was demonstrated by a dramatic increase in *Lactobacilli* and *Bifidobacteria*, reduction on the general count of *E. coli*, complete disappearance of the hemolytic *E. coli* and other pathogens such as *Staphylococci*, *Proteus vulgaris* and even *Candida* sps. In the remaining 15.5% of the patients, a significant improvement was observed.

Example 11

Application of the food supplement (Example 5) in patients with late radiation enterocolitis

The food supplement (10 ml, 3 times a day for 14 days, 30 min before the meals) was given to 24 patients with womb, colon and gastric cancer that developed enterocolitis following radiation therapy or a combination of radiation and chemotherapy.

Prior to the use of the food supplement, all patients complained about frequent and painful desire to defecate, liquid stool (4-12 times a day) appearance of mucous (9 cases) and blood (3 cases) in the stool.

Two or three days after ingesting the supplement, all the patients noted the lessening of pain and reduction in the number of defecations; the stool had a normal appearance. Four to five days later the diarrheal syndrome was gone, the appearance of blood and mucus ceased. The blood analysis showed a strong improvement in blood indicators.

In the control group of a similar size the symptoms persisted.

Example 12**Application of the food supplement (Example 4) in AIDS patients**

Patients suffering from AIDS frequently develop chronic diarrhea. A group of such patients was given 10 ml of the food supplement (Example 5) 3 times a day 30 min before the meals for 20 days. The control group received no supplement. The results of the treatment are shown in Table 4.

Table 4

Parameter	Food supplement	Control
No. of patients	30	20
Average age, years	38±1	36±2
Daily defecation frequency:		
at the onset of the Exp.	3.4±0.3	3.6±0.3
at the end of the Exp.	1.1±0.1	3.2±0.3
one month after the Exp.	1.5±0.2	3.7±0.3
Av. onset of normalization	6.0±0.7	remained abnormal

Patients receiving the food supplement showed normalization of intestinal microflora: reduction in general number of coliforms, disappearance of the hemolytic *E. coli*, increase in the numbers of *Lactobacilli* and *Bifidobacteria*, reduction in *Candida* sps.

Example 13**Application of the food/feed additive (Example 3) for accelerated weight increase in healthy piglets**

Healthy piglets were administrated 3 ml *per os* of the food/feed additive per piglet per day until weaning. The group receiving the feed additive gained

weight at weaning on the average 1.0 kg per piglet more than the control group.

Example 14

Application of the feed additive (Example 5) in healthy piglets

Several hundreds healthy piglets were administrated 3 ml *per os* of the feed additive per piglet on the first and third day after delivery and at weaning. Mortality was cut down by 50% compared with the control group receiving standard prophylactic treatment with antibiotics. The group receiving the feed additive gained weight at weaning on the average 0.39 kg per piglet more than the control group. When the feed additive and antibiotic treatment were compared in the same litter, the weight gain in the piglets obtaining the feed additive was found higher by 2.4 kg than in the control.

Example 15

Application of the feed additive (Example 4) in piglets showing diarrhea

Several hundred piglets showing diarrhea were given daily 5 ml *per os* of the feed additive per piglet. The control group of the same size was treated with antibiotics: advocin, gentiamycin, amoxicillin. The symptoms of diarrhea in the group receiving the feed additive disappeared within 1-2 days. No mortality was observed, and piglets developed normally. Antibiotics stopped diarrhea in the great majority of piglets but the piglets remained stunted in their development.

About 70 retarded piglets, that received antibiotic treatment against diarrhea for a week, and generally considered lost, were given the feed additive for three days. All but two survived.

Example 16**Application of the feed additive (Example 4) in healthy calves**

Day-old healthy calves were administrated 5 ml of the feed additive a day in milk during 7 days. More than 95% of the calves did not developed diarrhea until they were 14 days old, when a few cases positive for Rotavirus were diagnosed.

Example 17**Application of the feed additive (Example 6) in calves showing diarrhea**

Calves developing diarrhea were given daily 10 ml of the feed additive per animal with milk during 3-5 days. The symptoms of diarrhea disappeared within 1-2 days in 90-95% of calves. In the remaining 5-10% the diarrhea was caused by virus. These calves were treated with antibiotics with poor results.

Example 18**Application of the feed additive (Example 6) in healthy lambs and goat kids**

Day-old healthy lambs and goat kids were administrated 3 ml of the feed additive a day in milk during 7 days. In some cases slight to moderate signs of diarrhea were observed. These signs usually disappeared spontaneously or were successfully treated with an increased dose of the feed additive (5 ml).

Example 19**Application of the feed additive (Example 5) in lambs and goat kids showing diarrhea**

The feed additive was tested in a herd suffering from pathogenic *E. coli* infections. In the year preceding the experiment, about 90 from 120 lambs and goat kids died from diarrhea. Treatment with antibiotics was ineffective, since the disease developed suddenly and with fast mortality. Lambs and goat kids developing diarrhea were given daily 5 ml of the feed additive per animal with

milk during 3-5 days. A matching group of animals was treated, as normally recommended, with antibiotics. The symptoms of diarrhea disappeared within 1-2 days in about 90% of the lambs and goat kids receiving the feed additive. Their further development appeared normal. The control group receiving antibiotics (gentamycin) showed much poorer results. Diarrhea in this group persisted, calling for repeated treatment with antibiotics. The development of the control group was severely retarded.

Example 20

Application of the feed additive (Example 5) in poultry

The feed additive was added to the drinking water with the average uptake of 0.01 ml of the additive per day per chick during the breeding period (42-49 days). An increase of 3.2% in weight gain, accompanied by 4% improvement in food conversion was noted in controlled trials in broilers.

Excellent results were also obtained with turkeys of age 1 day to 6 weeks. Each bird received 0.01 ml of the additive per day. A weight gain of over 10% was observed, along with reduced mortality rate. Birds which still exhibited diarrhea, were treated with 0.1 ml per day of the food additive, without any treatment by antibiotics, and showed better recovery from the control birds which were treated with only antibiotics.

Example 21

Application of the feed additive (Example 5) in dogs and cats

Application of the feed additives in puppies resulted in cessation of the symptoms of diarrhea within 24-48 hours.

CLAIMS:-

- 1) *Escherichia coli* strain BU-230-98 ATCC Deposit No. 20226 (DSM 12799)
- 2) A formulation comprising at least one volatile fraction (VF) of a plant extract, said volatile fraction being prepared by steam distillation of said plant extract under reduced pressure and at a bath temperature not exceeding 38°C.
- 3) A formulation as claimed in claim 2, wherein said extract is obtained from the leaves, stems, roots or fruit of said plant.
- 4) A formulation as claimed in claim 2 or 3, wherein said plant is a vegetable or herb.
- 5) A formulation as claimed in claim 4, wherein said vegetable is soy bean, alfalfa, garlic, beet and cabbage.
- 6) A formulation as claimed in claim 4, wherein said herb is parsley, mint or dill.
- 7) A formulation as claimed in any one of claims 2 to 6, further optionally comprising propolis or other beehive product.
- 8) A veterinary feed additive for enhancing animal weight gain comprising a formulation as claimed in any one of claims 2 to 7.
- 9) A veterinary additive as claimed in claim 8, consisting of a formulation as claimed in any one of claims 2 to 7.
- 10) A veterinary additive as claimed in claim 8 or 9, comprising distilled water and volatile fractions of alfalfa, soy beans, beet and dill, preferably at a ratio of volatile fractions of 2:8:1:4 (v/v).

- 11) A probiotic composition comprising a formulation as claimed in any one of claims 2 to 7, and an effective amount of at least one viable probiotic microorganism having a beneficial biological or therapeutic activity in the gastrointestinal tract.
- 12) A probiotic composition as claimed in claim 11, wherein said probiotic microorganism is *E. coli*.
- 13) A probiotic composition as claimed in claim 8, wherein said probiotic microorganism is the *E. coli* strain BU-230-98, ATCC Deposit No. 202226.
- 14) A probiotic composition as claimed in any one of claims 11 to 13, for preventing or treating gastro-enteric infections.
- 15) A probiotic composition as claimed in claim 14, for the prevention or treatment of gastro-enteric infection caused by an enteric pathogen.
- 16) A probiotic composition as claimed in claim 15, wherein said pathogen is a Gram negative bacterium or Gram positive bacterium.
- 17) A probiotic composition as claimed in any one of claims 11 to 16, comprising distilled water and volatile fractions of alfalfa, soy beans, beet, dill and mint, preferably at a ratio of 5:1:5:15:1 (v/v).
- 18) A probiotic composition as claimed in any one of claims 11 to 17, for preventing or treating gastro-enteric *Salmonella* infection.
- 19) A probiotic composition as claimed in any one of claims 11 to 17, for preventing or treating infectious diarrhea, chronic diarrhea or diarrhea resulting from antibiotic therapy.
- 20) A composition as claimed in claim 19, for treating infectious diarrhea, comprising distilled water and volatile fractions of alfalfa, soy beans, beet, dill and mint, preferably at a ratio of 5:1:5:15:1 (v/v).

- 21) A composition as claimed in claim 20, wherein said infectious diarrhea is caused by *C. difficile*, *Salmonella*, particularly *S. Shigella*, *Campylobacter*, *E. coli*, *Proteus*, *Pseudomonas* or *Clostridium*.
- 22) A composition as claimed in claim 11, for effectively restoring the gastrointestinal microflora in a subject, thus normalizing the physiological activity of the gastrointestinal tract.
- 23) A probiotic composition for the normalization of the function of the gastrointestinal tract as claimed in claim 22, comprising distilled water and wherein said volatile fractions are volatile fractions of alfalfa, soy beans, beet, dill, mint, parsley and cabbage, preferably at a ratio of volatile fractions of 1:5:5:2:2:1, and said probiotic microorganism is the non-pathogenic *E. coli* strain BU-230-98, ATTC Deposit No. 202226.
- 24) A probiotic composition as claimed in claim 11, having an anti-tumor activity, capable of preventing the development of tumor cells and/or of suppressing growth of tumor cells.
- 25) A probiotic composition as claimed in claim 11, for treating dyspeptic symptoms.
- 26) A probiotic composition as claimed in claim 11, for stimulating the immune response in a patient suffering from an immune disorder.
- 27) A probiotic composition as claimed in claim 26, wherein said immune disorder results from immune-response suppression therapy.
- 28) A liquid formulation as claimed in claim 2, which has a long shelf-life.
- 29) A liquid probiotic composition as claimed in claim 11, which has a long shelf-life.
- 30) A process for preparing a volatile fraction of a plant extract, comprising the steps of:-

- a) grinding a plant or plant part to give a plant biomass;
 - b) mixing the biomass obtained in step (a) with water at a weight ratio of 3 parts water to 1 part biomass and stirring the same for at least 2 hours at ambient temperature;
 - c) steam distilling the mixture obtained in step (b) under reduced pressure and at a bath temperature not exceeding 38°C; and
 - d) collecting the volatile fraction obtained from said steam distillation, which fraction may be further diluted with a suitable buffer.
- 31) A process as claimed in claim 30 for preparing a formulation consisting of a volatile fraction of a plant extract, optionally further comprising mixing the volatile fraction obtained in step (d) with physiologically or veterinary acceptable carriers or diluents.
- 32) A process for preparing a formulation comprising at least one volatile fraction of a plant extract comprising mixing different plant extract volatile fractions as obtained in step (d) of claim 30, and optionally further comprising adding to the mixture physiologically or veterinary acceptable additives, carriers or diluents.
- 33) A process as claimed in any one of claims 30 to 32, wherein said plant extract is obtained from the leaves, stems, roots or fruit of said plant.
- 34) A process as claimed in claim 33 wherein said plant is a vegetable or herb.
- 35) A process as claimed in claim 34 wherein said vegetable is selected from soy, alfalfa, garlic, beet and cabbage.
- 36) A process as claimed in claim 34 wherein said herb is parsley, mint or dill.

- 37) A process as claimed in any one of claims 32 to 36 wherein said additive is propolis or other suitable beehive product.
- 38) A process as claimed in claim 30, wherein said distillation is performed at a reduced pressure of 5-10 mbar.
- 39) A process as claimed in any one of claims 30 to 32, further comprising the step of suspending at least one viable probiotic microorganism having a beneficial biological or therapeutic activity in the gastrointestinal tract in the volatile fraction obtained in step (d) of claim 30 or in said mixture of such volatile fractions obtained by the process as claimed in claim 32.
- 40) The process as claimed in claim 38, wherein said probiotic microorganism is the non-pathogenic *E. coli* strain BU-230-98, ATCC Deposit No. 202226.
- 41) Use of a volatile fraction (VF) of a plant extract prepared by steam distillation of said plant extract at a bath temperature not exceeding 38°C in the preparation of a food/feed additive.
- 42) A formulation comprising a volatile fraction of at least one plant extract, substantially as described in the specification.
- 43) A process for preparing a food/feed additive comprising at least one volatile fraction of a plant extract, substantially as described in the specification.
- 44) Use of a composition comprising at least one volatile fraction of a plant extract prepared by steam distillation of said plant extract at a bath temperature not exceeding 38°C, and a viable, non-pathogenic probiotic microorganism, in the preparation of a food/feed additive.

- 45) A process for preparing a probiotic composition comprising at least one volatile fraction of a plant extract and a viable, non-pathogenic probiotic microorganism, substantially as described in the specification.

INTERNATIONAL SEARCH REPORT

International application No
PCT/IL 00/00318

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N1/20 A61K35/74 A61K35/78 A61P1/00 A61P37/00
A23K1/00 A23L1/03 C12R1/19 //(C12N1/20,C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K A61P A23K A23L C12R C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, PAJ, WPI Data, FSTA, BIOSIS, CHEM ABS Data, CAB Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GB 1 501 098 A (BREWING PATENTS LTD) 15 February 1978 (1978-02-15)	2-4, 30-34, 38,41-43
Y	example 1 claims 1-7,12,13	8,9,11, 12, 14-16, 18,19, 21,22, 39,44,45

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Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

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L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

8 document member of the same patent family

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/IL 00/00318

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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